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Breakdown of paramylon and laminarin by digestive enzymes of Lamellibranchs — an important ecological feature

by

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§ 1. Introduction, and statement of the problem

In the low western part of the Netherlands, waterblooms composed almost entirely of Euglenids are not uncommon in some of the water-filled ditches whose combined length would cover almost two thirds of the distance to the moon. Paramylon, the β-1:3-linked glucan which forms the reserve polysaccharide of green (photosynthetic) as well as colorless Euglenids, may make up a considerable proportion of the dry weight of these blooms (up to 30-35% in certain cases). This polysaccharide is found in the cells in the form of microscopic granules, discs or rods, insoluble even in boiling water and displaying such a high degree of invulnerability towards chemical agents and enzymatic attack that, in an ecological sense, paramylon must be considered to be somewhat comparable to cellulose. Therefore, a logical question to ask is, whether the "fixed" carbon of paramylon can be mobilized again when the waterblooms die off; and, if so, under what circumstances and in what ways is this accomplished? In the present article, we shall consider the possibility that freshwater Lamellibranchs (common in the same areas where the Euglenids are found, at least if the water is not too brackish) play a role in the mobilization process. Thus, we have studied the action of lamelli-

branch stomach extracts on β-1:3-linked glucans. For purposes of comparison, however, we have included some enzymes from other sources in our investigations.

§ 2. MATERIAL AND METHODS

A. *Enzymes from various sources*

The quest for enzymes able to act on native paramylon was somewhat facilitated by the fact that laminarin, another β-1:3-linked glucan, is water-soluble; it can thus be substituted for paramylon in initial screening-experiments. Only those enzyme mixtures (extracts of organs, suspensions of bacteria, etc.) which are able to hydrolize laminarin vigorously can be expected to act on native (raw) paramylon. Furthermore, some help was provided by the circumstance that, at present, a great deal of attention is being paid to the distribution of "laminarases" in nature, in connection with the growing conviction that, in a quantitative sense, the β-1:3-linked glucans (which include certain reserve carbohydrates of diatoms; see, for this, VON STOSCH (1951, pp. 192-193), BEATTIE *et al.* (1961, pp. 531-537) and MEEUSE (1962, pp. 298-299; 1963, pp. 223-224)) may well be the most important carbohydrates on this planet. Thus, "laminarases" have now been studied in bacteria and fungi (see REESE & MANDELS, 1963, p. 208; CHESTERS & BULL, 1963, pp. 28-31), higher plants (CLARKE & STONE, 1960, pp. 175-188; REESE & MANDELS, 1963, p. 208 and table 2a) and, not surprisingly, in Euglenids themselves (FELLIG, 1960, p. 832; MEEUSE, 1962, p. 298; TOCHER, 1962, pp. 1-54). However, strange to say, the study of "laminarases" in invertebrates has been woefully deficient; specific information has been provided only by MEEUSE & FLUEGEL (1958 a, pp. 699-700; 1958 b, table 1), STONE & MORTON (1958, pp. 127-141), MYERS & NORTHCOTE (1958, pp. 639-648); QUILLET (1958, pp. 812-815), GALLI & GIESE (1959, pp. 415-440), OVERGAARD NIELSEN (1963, p. 1001) and KOOIMAN (1964, p. 199), while remarks on β-glucosidic activity in general are to be found in HOLDEN & TRACEY (1950, pp. 407-414), EVANS & JONES (1962, pp. 149-160), and HORIUCHI (1963, pp. 133-152).

In the present study, enzymes from the following sources have been tried:

1. MOLLUSCA.

Anodonta piscinalis (Nilss.)
Anodonta cygnea zellensis (Gmel.)
Unio pictorum (L.)
Unio tumidis Philipsson
Dreissena polymorpha (Pall.)

Mya arenaria L.
Mytilus edulis L.
Margaritifera margaritifera (L.)
Cryptochiton stelleri Middendorff
Helix pomatia L.

In the case of *Cryptochiton,* the freeze-dried juice from the so-called sugar gland was used; it was redissolved in dilute phosphate buffer, pH 5.2. In the case of the snail *Helix pomatia,* fresh, clear hepato-pancreas juice from the crop was employed. The Lamellibranch enzymes were obtained by dissecting out the glands surrounding the stomach, grinding them with some quartz and dilute phosphate buffer (pH 5.2) in a mortar, and centrifuging the resulting mixture until the supernatant was fairly clear. No special effort was made to exclude portions of the crystalline style from our preparations.

2. CRUSTACEA.

Astacus leniusculus Dana, fresh stomach juice.

3. FUNGI.

Sporotrichum pruinosum Gilman et Abbott
Rhizopus arrhizus Fischer
Sclerotinia libertiana Fuck = *Scler. sclerotiorum* (Lib.) de Bary

In the case of *Sclerotinia,* aqueous extracts of a mold bran were used; in the other two cases, culture fluids.

4. BACTERIA.

Aerobacillus polymyxa (Prazmowski) Donker; grown on a medium composed of tap water, 0.5% peptone, 0.2% K_2HPO_4, 0.1% laminarine, and 0.4% fructose. In addition, mixtures of bacteria were used that were isolated from similar media inoculated with garden soil.

5. HIGHER PLANTS.

Ficin (commercial preparation)
Bromelin (commercial preparation)
Hordeum sativum L. (aqueous extract of germinating barley)

6. EUGLENIDS

(used for their enzymes as well as for the preparation of paramylon)
Astasia longa Pringsheim
Euglena acus Ehrenberg
Euglena deses Ehrenberg
E. gracilis Klebs var. *bacillaris* Pringsheim

E. gracilis Klebs strain Z
E. gracilis Klebs, apochlorotic form
E. limnophila Lemm.
E. oxyuris Schmarda
E. spirogyra Ehrenb.
E. tripteris (Duj.) Klebs
E. viridis Ehrenb.
Khawkinea ocellata var. *Provasolii* Pringsheim (formerly known as *Astasia ocellata* Khawkine)
Khawkinea pertyi Pringsheim
Phacus pleuronectes (O. F. M.) Duj.

B. *Paramylon.*

In order to obtain paramylon granules of a relatively large size (8-12 μ, occasionally up to 15 μ), *Phacus pleuronectes* (O. F. M.) Duj. was grown in soil/water cultures in moderate, diffuse light. The individuals were concentrated with the aid of the concentrator described elsewhere (MEEUSE, 1963b, pp. 423-424), after which they were killed and extracted with strong ethanol. Their cell-walls were eliminated by a 48 hr. digestion with trypsin in an aqueous medium (pH 7.5; phosphate buffer), and their paramylon granules, accompanied by much contaminating material, spun down in the centrifuge. They were now washed several times by resuspending them in distilled water and spinning them down again. Finally, the suspension of paramylon granules was acidified with some glacial acetic acid and shaken with butanol. The resulting emulsion, after standing for some time, separated into two layers: an upper butanol layer, containing most of the contaminating compounds, and a lower, aqueous layer, from which a fairly pure paramylon settled out. This procedure was repeated two or three times, yielding a snow-white, pure paramylon which was dried in an oven at 65° C. Under the light microscope, most of the paramylon grains turned out to be double granules, having roughly the shape of a yoyo or an American hamburger bun; in surface view, they were practically round or short oval, with a central hole (fig. 1, left). Between crossed Nicol prisms, the granules displayed a black cross, as is the case with starch granules. They differ from the latter, however, in having a helical structure in stead of a concentric one (see, for this, MEEUSE, 1963a, fig. 1). Thus, the surface view of a *Phacus* paramylon grain is very similar to the corresponding view of a *Planorbis* shell (fig. 1 of this article, left).

For purposes of comparison, paramylon granules were also isolated (in the manner described) from the 3 colorless forms *Astasia longa,*

Khawkinea ocellata and *K. pertyi*, and from the 6 green *Euglena* species (*E. acus, E. gracilis, E. limnophila, E. oxyuris, E. spirogyra* and *E. tripteris*). It is well-nigh certain that the granules of all these forms possess the helical structure. In shape, they range from almost solid ellipsoids (*E. gracilis, A. longa*) to more or less rectangular "chain-links" (*E. oxyuris*) and slender rods lacking a lumen almost entirely (*E. limnophila, E. acus*).

C. *Enzymatic breakdown of β-1:3-glucans.*

The breakdown of laminarin was followed chemically, at 30° C, in mixtures of the following composition: 0.5 ml of laminarin solution, containing 10 mg of the carbohydrate (in water); 0.5 ml enzyme solution; 0.5 ml M/15 phosphate buffer, pH 5.2; 1 ml water. Samples were withdrawn at regular intervals and sugar formation was investigated with the aid of filter paper chromatography and/or with a dinitrosalicylic acid reagent for measuring reducing power (see, for this, BERNFELD, 1955, pp. 149-150). For the filter paper chromatography, Whatman No. 1 filter paper was used in conjunction with one of the following running-fluids:

1) butanol/acetic acid/water 4:1:5 (upper phase);
2) butanol/pyridine/water 6:4:3;
3) butanol/pyridine/water/benzene 50:30:30:4.5;
4) n-propanol/ethylacetate/water 6:1:3;
5) ethylacetate/acetic acid/water 9:2:2.

(All the ratios given are volume/volume ratios).

As reference mixtures on the chromatograms, partial acid hydrolizates of laminarin were used which, in addition to β-1:3-linked compounds, demonstrably contained the β-1:6-linked disaccharide, gentiobiose. The above mixture No. 5, when used for 5 days with Whatman No. 1 filter paper at 25° C (descending method) always gave excellent separation of gentiobiose from the other sugars present.

Reducing sugars on the chromatograms were demonstrated either by a treatment with acetonic $AgNO_3$ followed by alcoholic KOH, or with a solution of anisidine in phosphoric acid; in the latter case, observation in ultraviolet light facilitated the interpretation vastly. The breakdown of paramylon was followed microscopically at 30° C by incubating a few mg of native (raw) paramylon with about 1 ml of enzyme/buffer mixture (pH 5.2) and a few drops of toluene as a disinfectant; at regular intervals, drops were removed for microscopic observation.

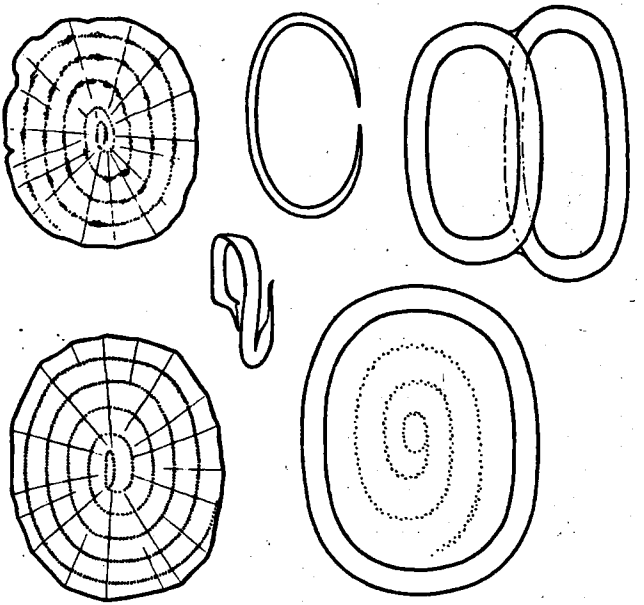

Fig. 1. Various stages in the enzymatic breakdown of native paramylon granules by molluscan laminarases. Lower left, intact *Phacus pleuronectes* granule showing spiral structure and small central hole. Upper left, slight etching of a *Phacus pleuronectes* granule by crop juice of *Helix pomatia*. Middle and right: formation of rings of astounding regularity by enlargement of the small central hole originally present.

§ 3. RESULTS

A. *Enzymatic breakdown of native paramylon.*

In general, even preparations that were extremely active towards laminarin (such as crayfish stomach juice, which contained the strongest laminarase ever encountered by us) were powerless when confronted with native paramylon. *Helix* crop juice and *Cryptochiton* sugar gland juice exerted only a slight etching action (Fig. 1, upper left); stomach extracts of *Mytilus* and *Mya* were somewhat active, all the other preparations inactive. Notable exceptions to this, however, were stomach extracts of the *Anodonta* and *Unio* species and of *Dreissena polymorpha*. Even here, the results were somewhat erratic. In the best cases, the roundish, disc-shaped paramylon bodies of *Phacus pleuronectes* were gradually transformed into slender hoops, while the "chainlinks" so characteristic of (e.g.) *Euglena oxyuris* would change into "paper clips" (Fig. 1). Whether the effect is due

to true molluscan enzymes or to that of symbiotic micro-organisms, it probably represents a dramatization or speed-up of events which may also take place in the normal, living *Euglena* cell. The occurrence of paramylon rings in *Euglena* cells should then not be ascribed to the existence of predetermined similarly shaped protoplasmatic structures, but to a lytic, enzymatic process. There is no evidence whatsoever for the existence of a membrane around paramylon bodies, and this makes the extreme regularity and the centrifugality of the breakdown pattern even more difficult to interpret. However, the observations become somewhat easier to explain when it is kept in mind that there is a very strict orientation of the micelles (and, hence, of the molecules) within the granule, as evidenced by the dark

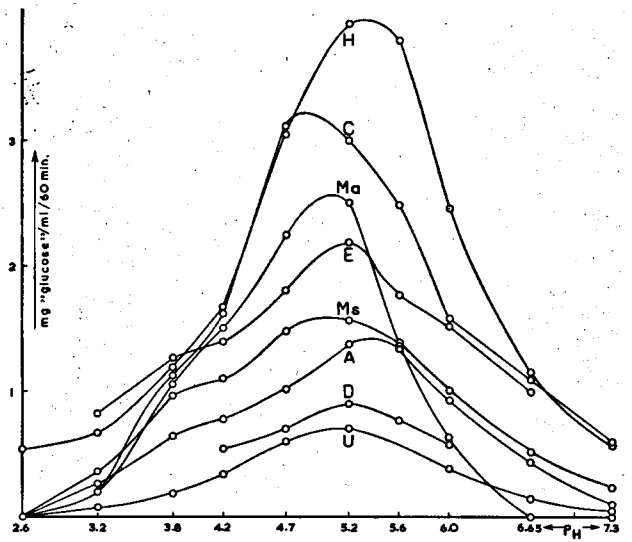

Fig. 2. pH-optimum of various molluscan laminarases and of *Euglena* laminarase. A = *Anodonta;* C = *Cryptochiton;* D = *Dreissena;* E = *Euglena;* H = *Helix;* Ma = *Mya;* Ms = *Mytilus;* U = *Unio.*

cross appearing between crossed nicol prisms (see above). Furthermore, from the present author's work on the chemistry of paramylon (MEEUSE, 1964, in press), it follows that the paramylon molecules possess great internal uniformity. Finally, it is not far-fetched to entertain the notion that certain enzymes work in a unidirectional fashion; a precedent is provided by β-amylase from malt, which attacks the terminal branches of amylopectin molecules by moving towards the branching-points.

B. *Some properties of the Lamellibranch laminarases.*

With a view to the considerations just given, it became imperative to characterize the laminarase enzymes present in the Lamellibranch stomach extracts, and to study their mode of action. Fig. 2 gives the pH-optimum for laminarase activity on the basis of measurements of reducing power with the dinitrosalicylic acid method. The amazing result is that, with the possible exception of *Cryptochiton,* whose laminarase seems to work best between pH 4.7 and 5.2, the optimum is at 5.2 for all the organisms tested, including *Euglena gracilis.* There is reasonable activity between pH 3.2 and 7.3. Somewhat exceptional was *Mya,* where the laminarase activity at pH values higher than 5.2 fell off more steeply than it did in the other cases, becoming practically zero at pH 6.65.

The temperature-optimum, like the pH-optimum, did not exhibit significant differences from one organism to the other; in experiments extending over a one-hour period, it was found to be at the amazingly high value of 55-60° C (Fig. 3).

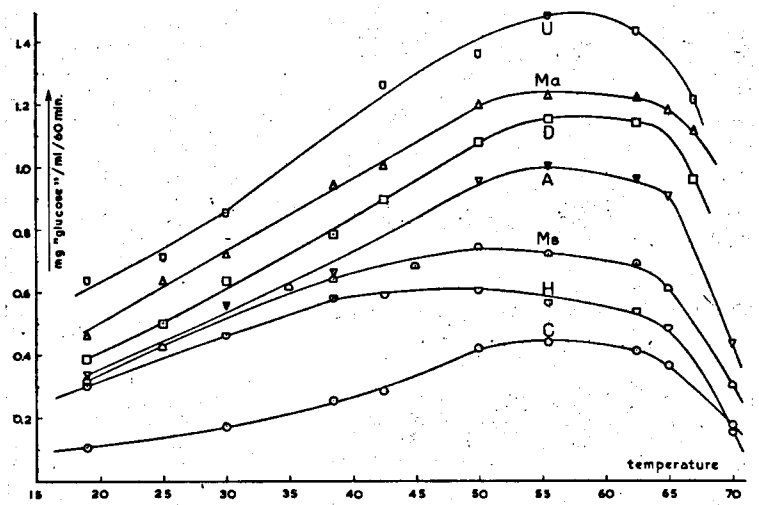

Fig. 3. Temperature optimum of various molluscan laminarases. Meaning of symbols as in Fig. 2.

The microscopic image of the enzymatic breakdown of native paramylon granules is compatible with the possibility that the molluscan laminarases act as "exolaminarases", eliminating from the

large paramylon molecules a succession of glucose molecules that are, presumably, terminal. For *Euglena* laminarase (with laminarin as the substrate), both FELLIG (1960, p. 832) and TOCHER (1962, p. 44) did indeed find glucose as the sole breakdown product. In the case of the molluscan enzymes, however, filter paper chromatography shows beyond any possible doubt that they act as "endo-laminarases", giving rise to a mixture of oligosaccharides in the initial phases of breakdown. (MEEUSE & FLUEGEL, 1958 a, pp. 699-700 and 1958 b, table 1 had already shown this to be the case for *Cryptochiton*). As an example, we shall quote the following cases (the dilution of the enzymes used in the experiments was arbitrary):

Unio-enzyme. After 80 minutes, spots for glucose, laminaribiose, laminaritriose, laminaritetraose, plus a trace of gentiobiose.

Helix-enzyme. After 15 minutes, the homologous series glucose—laminaribiose—laminaritriose—laminaritetraose and (probably) laminaripentaose. The following table, in which the arabic numbers indicate the number of 15-min. periods elapsed, summarizes the results over a longer time-span. Numbers between brackets indicate trace-amounts.

Spot No.	I	(glucose) (1); 2-12; 1 day.
„ „	II	(laminaribiose) (1); 2-11; (12); (1 day).
„ „	III	(gentiobiose) 5-12; (1 day).
„ „	IV	(laminaritriose) 1-4.
„ „	V	(probably gentiobiosyl-glucose) (4); 5-8.
„ „	VI	(laminaritetraose) 1,2.
„ „	VII	(probably laminaripentaose) 1-7; (8).

Anodonta-enzyme.

Spot No.	I	(glucose) 4-12.
„ „	II	(laminaribiose) 5-12.
„ „	III	(gentiobiose) 7-12.
„ „	IV	(laminaritriose) 5-12.
„ „	V	?
„ „	VI	(laminaritetraose) 7-12.

Cryptochiton-enzyme.

Spot No.	I	(glucose) 1-12; 1 day.
„ „	II	(laminaribiose) 1); 2-12.
„ „	III	(gentiobiose) 7-12.
„ „	IV	(gentiobiosyl-glucose?) 7-12.
„ „	V	(laminaritetraose) (1); 2-12.
„ „	VI	(laminaripentaose?) (2); 3-12.
„ „	VII	(laminaritriose) (1); 2-12.

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SUMMARY

A quest for enzymes or enzyme mixtures capable of attacking paramylon, the insoluble and chemically inert reserve polysaccharide of the Euglenids, in its raw state, has yielded positive results only for the stomach extracts of certain freshwater Lamellibranchs. These juices converted the doughnut-shaped native paramylon granules to slender rings of astounding regularity. This can be considered as a dramatization of the lytic events taking place during the mobilization of paramylon bodies in living Euglenid cells and giving rise to slender rings there; it militates against the idea that paramylon rings in Euglenid cells are produced on, or in, preformed protoplasmic structures of the same shape. Crop juice of the snail *Helix pomatia* and sugar gland juice of *Cryptochiton stelleri* exerted only a slight etching effect on paramylon granules. Stomach juice of crayfish (*Astacus*) and preparations from several species of bacteria, molds and higher plants were found to be inactive, although all of them (like the Lamellibranch enzymes and those from *Helix* and *Cryptochiton*) were very active towards dissolved laminarin. For all the animal laminarases tested, including the *Euglena* enzyme, the pH-optimum was close to 5. In experiments of 1 hr. duration, the temperature optimum for all the molluscan enzymes was remarkably high, namely 55-60° C. Filter paper chromatography revealed that, with the exception of the *Euglena* enzyme, the animal enzymes act as "endo-laminarases", giving rise to a mixture of several oligosaccharides before their substrate (laminarin) is ultimately converted to glucose.

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SAMENVATTING

Pogingen tot het vinden van enzymen of enzymmengsels die in staat zijn om paramylon, het in water onoplosbare en chemisch inerte reservekoolhydraat van de Eugleniden, in zijn natieve toestand aan te tasten, zijn vruchteloos gebleven met als enige uitzondering de maagenzymen van bepaalde zoetwater-lamellibranchiaten. Deze zetten de natieve paramylonkorrels, die de gedaante hebben van Amerikaanse „doughnuts", om in slanke ringen van een verbazingwekkend regelmatige structuur. Dit proces kan worden beschouwd als een dramatisering van de lytische processen die plaats grijpen tijdens het mobiliseren van paramylon-lichaampjes in de levende Euglenidencel en ook daar leiden tot de vorming van dunne ringen; het pleit sterk tegen de opvatting dat paramylon-ringen in de levende cel worden gevormd op, of in, gepraeformeerde protoplasmatische structuren die dezelfde gedaante hebben. Kropsap van de wijngaardslak *Helix pomatia* en sap uit de zg. suikerklier van *Cryptochiton stelleri* oefenden op paramylonkorrels alleen maar een zwakke etsende werking uit. Maagsap van zoetwaterkreeften (*Astacus*) en preparaten bereid uit verschillende soorten bacteriën, schimmels en hogere planten werden inactief bevonden, hoewel al deze preparaten (evenals trouwens de enzymen uit de Lamellibranchiaten, *Helix* en *Cryptochiton*)

zeer actief waren ten opzichte van opgelost laminarine. Voor alle dierlijke laminarasen die onderzocht werden, met inbegrip van het enzym uit *Euglena,* lag het pH-optimum dicht bij 5. In proeven die 1 uur duurden lag het temperatuur-optimum voor al de enzymen uit mollusken merkwaardig hoog, namelijk bij 55-60° C. Papierchromatographie bracht aan het licht dat, met uitzondering van het enzym uit *Euglena,* de dierlijke enzymen als „endo-laminarasen" werken, hetgeen zeggen wil dat hun werking leidt tot het ontstaan van een mengsel van oligosacchariden voordat hun substraat (laminarine) uiteindelijk is omgezet tot glucose.